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SCIENCE DIRECT
Journal of Virological Methods 134 (2006) 197–204
Journal of Virological Methods
www.elsevier.com/locate/jviromet

A long distance RT-PCR able to amplify the *Pestivirus* genome

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Received 23 June 2005; received in revised form 3 January 2006; accepted 10 January 2006
Available online 23 February 2006

Abstract

A method to amplify long genomic regions (up to ~12.3 kb) from pestiviruses in one RT-PCR is described. The difficulty in designing conserved *Pestivirus* primers for the amplification of genomes from highly divergent isolates simply by means of overlapping segments is demonstrated using new bioinformatic tools. An alternative procedure consisting of optimizing the length of the genomic cDNA fragments and their subsequent amplification by polymerase chain reaction (PCR) using a limited set of specific primers is described. The amplification of long DNA fragments from a variety of sources, including genomic, mitochondrial, and viral DNAs as well as cDNA produced by reverse transcription (RT) has been achieved using this methodology, known as long distance PCR. In the case of viruses, it is necessary to obtain viral particles from infected cells prior to RT procedures. This work provides improvements in four steps of long distance RT-PCR (L-RT-PCR): (i) preparation of a viral stock, (ii) preparation of template RNA, (iii) reverse transcription and (iv) amplification of the cDNA by LD-PCR. The usefulness of L-RT-PCR is discussed in the light of current knowledge on pestivirus diversity. The genomic sequence of *Singer_Arg* reference strain obtained using this method is presented and characterized.

Keywords: Pestivirus; L-RT-PCR; *Flaviviridae*

1. Introduction

Pestiviruses are important livestock pathogens responsible for significant economic losses world-wide (Rice, 1996; Houe, 1999). The presence of bovine pestiviruses in cell cultures and in fetal bovine serum has been recognized as a relevant problem not only in research laboratories but also among biological manufacturers (Nutall et al., 1977).

The genus *Pestivirus* belongs to the family *Flaviviridae*. *Flaviviridae* also includes *Hepacivirus* (*Human hepatitis C virus*, HCV) and *Flavivirus*. There are two *Pestivirus* species that primarily infect bovines: *Bovine viral diarrhea virus 1* (BVDV 1) and *Bovine viral diarrhea virus 2* (BVDV 2) (Paton et al., 1995; Becher et al., 1999). Other members of *Pestivirus*, *Classical swine fever virus* (CSFV) and *Border disease virus* (BDV), infect pigs and sheep, respectively (Paton et al., 1995; Becher et al., 1999). Two new species of *Pestivirus* have been recently proposed, each with only one member: Giraffe Isolate and Reindeer Isolate (Avalos-Ramirez et al., 2001). Pestiviruses possess a single stranded positive sense RNA genome with a length of 12.3 kb.

Recent studies have shown that BVDV 1 may include more than 12 genotypes (Vilček et al., 2001; Jones et al., 2004). Each viral genotype seems to cause different clinical manifestations (Baule et al., 1997, 2001; Jones et al., 2001, 2004; Fulton et al., 2002). When this report was written, there were only six complete *BVDV 1* genomic sequences available in public sequence databases.

Full genome sequencing is used in several areas of virology, from taxonomy and phylogeny (e.g. Herniou et al., 2001; Avalos-Ramirez et al., 2001) to viral molecular biology (e.g. Kümmerer et al., 1998, 2000; Becher et al., 1996). Traditional sequencing strategies can be cumbersome and time consuming due to library construction and screening processes, developing of overlapping RT-PCR reactions and problems related to DNA sequence automation.

Reverse genetics permits the use of cDNA copies of viral RNA genomes to produce detailed studies of molecular features of virus infection, replication, and assembly. For pestiviruses, the availability of full-length cDNAs has relied on laborious

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doi:10.1016/j.jviromet.2006.01.005

genetic engineering techniques (e.g. Meyers et al., 1996). Furthermore, assembling cDNA clones obtained independently into a full-length sequence can lead to the combination of segments corresponding to different quasispecies.

In the present report, it is shown that finding conserved regions for designing a unique set of RT-PCR primers capable of amplifying many short overlapping cDNA fragments from isolates of different filiation might be impossible. For this reason, an alternative method which is able to amplify long RNA spans was developed. Previous reports have described the amplification of long DNA templates from eukaryotic genomes or molecular clones (Cheng et al., 1994). LD-PCR methods capable of amplifying long viral DNA genomes have been described (Barnes, 1994). Complete double stranded genomic RNA fragments of 0.8–6.8 kb have been successfully amplified (Potgieter et al., 2002). Complete genomes and long genomic regions of up to 10 kb long from other positive stranded RNA viruses have been reverse transcribed and amplified by PCR (Lindberg et al., 1997; Lindberg and Andersson, 1999; Holterman et al., 2000; Lu et al., 2005; Zhang et al., 2001). Long fragments of up to 20 kb from coronavirus genomic RNA have been successfully amplified by using a purification step previous to the RT (Thiel et al., 1997). This success strongly suggested the possibility of amplifying the whole pestiviral genome directly from total RNA; that is, avoiding viral RNA purification procedures.

To our knowledge, there have not been previous attempts to amplify complete *Pestivirus* genomes in vitro. In this report, it is shown that viral genomic RNA spans of up to 12.3 kb can be efficiently amplified by RT followed by LD-PCR directly from total RNA obtained from infected cells. The use of this technique is discussed in the light of current knowledge about pestiviral diversity. The genomic sequence of one of the reference strains used at the authors' laboratory is presented and characterized.

2. Materials and methods

2.1. Virus culture

The cytopathic (CP) *BVDV 1 Singer* reference strain used at the authors' laboratory (*Singer_Arg*) was propagated at low (0.01 plaque forming unit/cell) multiplicity of infection (MOI) in MDBK cells grown in minimal essential medium (MEM) supplemented with irradiated fetal bovine serum (FBS). To release all viral particles, cells were freeze–thawed once. At least three passages in cell culture at low MOI were performed.

For plaque forming unit assays, MDBK cells were grown on six wells plates (750,000 cells per well) and inoculated with 500 μl of 1:10 serial dilutions of virus suspensions. After incubating at 37 °C for 30 min, the cells were washed with PBS preheated at 37 °C and covered with 2.5% Methyl cellulose/MEM supplemented with 5% FBS. About 72 h later, the monolayers were fixed with 4% formalin and stained with 0.1% *Crystal Violet*.

Strains *T1*, *2B, 36P* and *66_6* were propagated following standard procedures described elsewhere (Jones and Weber, 2001; Jones et al., 2001).

2.2. Total RNA purification and cDNA synthesis

Total RNA from infected cells was extracted using a commercial reagent (Trizol, Promega). Aliquots of 150 μl of virus suspensions were added to 850 μl of Trizol. Manufacturer instructions were slightly modified, as 1 μg of yeast tRNA was added to the mixture prior to the organic extraction phase. Yeast tRNA was prepared at a concentration of 10 mg/ml in a solution of 10 mM Vanadyl Ribonucleoside in DEPC-treated double distilled water. Yeast tRNA was diluted 1:10 in ultra pure water (*GIBCO*) at the moment of being used. RNA pellets were resuspended in 5 μl of ultra-pure water and immediately used for RT.

For cDNA synthesis, 1 μl of 2 pM specific primer, 1 μl of 25 mM dNTPs, 6 μl of ultra-pure water (*GIBCO*) and 5 μl of RNA suspension were mixed and heated at 65 °C during 6 min. The preparation was chilled on ice for 1 min. Next, 4 μl reverse transcriptase buffer (provided by the manufacturer), 1 μl of 0.1 M DTT, 1 μl (40 U) *RNasin* ribonuclease inhibitor (*Promega*) and 200 U (1 μl) *Superscript III RNase*$^{(-)}$ *Reverse Transcriptase* (*Invitrogen*) were added. The mixture was incubated for 1 h at 55 °C; after which enzyme inactivation was carried out at 70 °C for 15 min. cDNA solutions were immediately used or stored at −20 °C. The performance of reverse transcription protocols published elsewhere (Jones and Weber, 2001; Jones et al., 2001) was also investigated.

2.3. LD-PCR

The amplification of cDNA targets longer than approximately 1 kb requires removal of complementary RNA. In order to achieve this, RNA/cDNA hybrids were treated by adding Na(OH) to a final concentration of 0.1N, or with 2 U of Ribonuclease H (Invitrogen). In both cases, samples were incubated for 20 min at 37 °C.

PCR reactions were carried out in 50 μl volume, using 2.5 U of *AccuPrime Taq DNA Polymerase High Fidelity* (*Invitrogen*), *Buffer I* (Provided by the manufacturer; 10× buffer: 600 mM Tris–SO_4 (pH 8.9), 180 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, 2 mM dGTP, 2 mM dATP, 2 mM dCTP, 2 mM dTTP, 10% glycerol, thermostable AccuPrime^{TM} protein), 2 μl cDNA obtained as described above, and a final concentration of 0.5 μM of each PCR primer. LD-PCR cycling profiles were: 15 s at 94 °C, followed by 35 cycles of 15 s at 94 °C, 15 s at 55 °C and 7–17 min (depending on the template's length) at 68 °C; these cycles were followed by a final extension period of 10 min at 68 °C. LD-PCR protocols published elsewhere (Barnes, 1994; Cheng et al., 1994) were also analyzed as described in Section 3.4. All the reactions were performed on a Perkin-Elmer 2400 thermocycler.

Aliquots of the LD-PCR amplification products were analyzed on 0.8% agarose standard horizontal gels. DNA was stained with 0.5 μg/μl ethidium bromide. Molecular markers (λ DNA digested with *Hin*dIII and/or 1 kb *DNA ladder* from Invitrogen) and precision molecular mass standards (Bio-Rad) were included when needed.